Human Leukocyte Antigens class II influence the expression of Glutamic Acid Decarboxylase auto antibodies in Type Diabetic children and their Siblings

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Abstract:

Background: The immunogenetic predisposition may be considered as an important factor for the development of Type 1 Diabetes Mellitus (T1DM) in association with the HLA antigens.

Objective: This study was designed to investigate the role of HLA-class II antigens in the etiology of type T1DM and in prediction of this disease in siblings, and its effect on expression of glutamic acid decarboxylase autoantibodies (GADA).

methods: Sixty children who were newly diagnosed type 1 diabetes (diagnosed less than five months) were selected. Their age ranged from 3-17 years. Another 50 healthy siblings were available for this study, their ages range from 3-16 years. Eighty apparently healthy control subjects, matched with age (4-17) years, sex and ethnic backgrounds (Iraqi Arabs) underwent the HLA-typing examination. Finally 50 healthy individuals were selected randomly to undergo GADA test.

Results: At HLA-class II region, DR3 and DR4 were significantly increased in patients (53.33 *vs*.26.25% and 50.0 *vs.* 12.5% respectively) as compared to controls. In addition to that, T1DM was significantly associated with DQ2 (33.33 *vs*.15%) and DQ3 (40.0 *vs*.20%) antigens as compared to controls, suggesting that these antigens had a role in disease susceptibility, while the frequency of DR2 and DQ1 antigens were significantly lowered in patients compared to controls (6.66 *vs*.25% and 6.66 *vs*.22.5% respectively). These molecules might have protective effect. In siblings a significant increase frequency of DR4 antigen (34.0 *vs*.12.5%) was observed in comparison to controls, suggesting that it might be much useful for predicting T1DM in affected families.Anti-GAD autoantibodies were present in 50% of Type 1Diabetic children, and in 16% of their siblings. High proportion of GADA was found in the patients carrying HLA-DR3/DR4 heterozygous.

conclusion: Both the T1DM patients and their siblings shared the HLA- DQ1 as protective antigens, while DR3 and DR4 were susceptible one, and high proportion of GADA was found in the T1DM patients and siblings carrying HLA-DR3/DR4 heterozygous.

Key words: T1DM patients, Siblings, HLA class II, GAD auto antibodies.

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Introduction:

The importance of genetic factors in the etiology of type 1 diabetes mellitus (T1DM) is demonstrated by the concordance rate of 5-10% in dizygotic twins, and up to 27% in monozygotic twins (1). Human Leukocyte Antigen (HLA) genes contribute up to 40% of familial aggregation of T1DM. Distinct loci within the HLA region determine risk, though the HLA class II region appears to be most influential. Protective alleles, haplotypes, and genotypes have been identified also (2). Antibody screening of first- degree relatives of T1DM patients suggests that antibodies to insulin are the first to appears, while Glutamic acid decarboxylase (GAD65) and protein tyrosine phosphatase- related (IA-2) auto antibodies may follow. Within two years of age, over 10% of first- degree relative infants appear to have at least one islet antibody, suggesting that the autoimmune process is initiated early (3, 4).

The frequency of GAD auto antibodies has been reported to vary from 0.5 - 3% among children from background population (5), from 6.4 - 13% among siblings of children with T1DM (6), and from 62 - 84% among patients with newly diagnosed disease (7).

GADA has been reported to be associated with the DQ2 alleles (8), and DR3.DQ2 haplotypes (9). During normal immune responses, molecules encoded by DR&DQ genes bind and present peptide fragments of

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protein antigens to lymphocytes of the CD4 subset. These class II molecules could play a pivotal role in the development of T1DM through presentation of islet-cell specific peptides to autoimmune CD4 T lymphocyte (10). Most studies assessing the influence of human leukocyte antigen on T1DM have analyzed population of individuals with known T1DM. We aimed to analyze the influence of HLA class II antigens on GAD65 expression on T1DM patients and first-degree relatives with positive auto antibodies.

Methods:

Subjects:

Sixty Iraqi Type 1 diabetic patients (28 males and 32 females) were subjected to this study. The patients were attending the National Diabetes Center at Al-Mustansiriya University during the period May 2004 to October 2005. Their ages ranged from 3 -17 years, and they were new onset of the disease (diagnosis was from one week up to five months). Diagnosis of Diabetes Mellitus and selection of patients was accomplished with the assistance of the consultant medical staff in the National Diabetes Center. All the patients were treated with daily replacement doses of insulin at the time of blood sampling. Fifty healthy first degree siblings (25 males and 25 females) of type 1 diabetes patients were underwent the study. Their ages ranged from 3 – 16 years. For the purpose of comparisons, 80 apparently healthy control subjects matched for age (4-17 years old), sex and ethnic back ground (Iraqi Arabs) were selected as a control group for HLA typing who had no family history of diabetes. Inflammatory diseases were ruled out in this group based on medical history, physical examination, and negative C- reactive protein. Out of these 80 controls, 50 healthy subjects (25 males and 25 females) were randomly selected for investigation of GAD_{65} auto antibodies.

All the participants gave their informed constant before being included in the study.

Collection of Blood Samples:

Ten milliliter

of venous blood was collected from each patient, sibling, as well controls. Eight milliliter of blood was put in heparinised test tube (10 U/ml) used for lymphocyte separation for the detection of HLA polymorphism. The remaining two milliliter was collected into plain test tube, and then the serum was separated by centrifugation and kept at -20°C for the assessment of GAD_{65} auto antibodies.

Serological Typing of HLA Antigen:

Lymphocytes were separated from the whole blood using Ficoll- Isopaque density centrifugation (Flow-Laboratories, UK) that was reported by Schendel *et al.,* (11). The collected cells were resuspended in 2 ml warm RPMI-1640 (Euroclone, UK) supplemented with 10% heat inactivated human type AB serum **(**National blood transfusion center**).** The lymphocyte population was separated by nylon wool method to T- cells and B- cells. B-lymphocytes were used for phenotyping of class II (DR and DQ) antigens (12). Cells were counted and determined their viability.

HLA class II typing –DR and –DQ locus was performed by conventional HLA serology (13). This test was carried out in the histocompatibility laboratory at the Al-Karama hospital.

Assessment of serum anti- GAD_{65} a utoantibodies:

Serum GADA was measured by Immunoradiometric assay (IRMA) using anti-GAD IRMA kit (Immunotech Beckman Coulter).

Statistical analysis:

Regarding of HLA and disease association the frequency distribution for selected variables was done first. The strength of disease association with particular HLA antigen was determined by calculating the relative risk (RR). A RR value can range from less than one (negative association) to more than one (positive association) while RR value of 1 indicates no differences in disease susceptibility. If the association is

negative, it indicates a protective effect, therefore the preventive fraction (PF) was calculated, while if it is positive, it indicates increased susceptibility to that specific disease, therefore the etiological fraction (EF) was calculated (14). The significance of such association was assessed by Fisher exact probability. Chi square tests were used for the measurements of correlation and dependency among different variable observations.

Results:

HLA Antigen Association

This test was conducted on the following main groups: Sixty T1DM patients, eighty healthy controls and fifty siblings. The frequencies of HLA antigens class II (-DR; and -DQ) were compared between T1DM patients and controls, siblings and controls and between T1DM patients and siblings.

HLA association with T1DM:

The distribution of HLA-DR; and -DQ antigens with their frequencies in T1DM patients and controls are presented in table (1), while antigens showing significant variations between patients and controls are given in table (2). At HLA-class II region (DR-loci), three antigens showed different frequencies in patients and controls, these were DR2, DR3, and DR4. Increased frequencies of DR3 (53.33 *vs.* 26.25%) and of DR4 (50.00 *vs.* 12.50%) were observed in patients. The positive association RR values were of 3.210 and 7.00 respectively and EF values of 0.366 and 0.428 respectively. Such positive association was highly significant ($P=9.7x10^{-3}$ and $1x10^{-5}$ respectively) and remained highly significant after correction (PC=0.008 and $9x10^{-5}$ respectively). In contrast DR2 antigen significantly decreased in the patients (6.66 *vs.* 25.00%) with PF value of 0.195. Such negative association was significant (P=0.003) and remain significant after correction (PC=0.027).

At HLA-DQ loci, two antigens DQ2 and DQ3 were significantly increased in the patients compared with controls (33.33 *vs.* 15.00%, P=0.009, RR=2.833, EF=0.215) for DQ2 while (40.0 *vs.* 20.0%, P=0.008, RR=2.666 and EF=0.249) for DQ3. This positive association remained significant after correction (PC=0.027 and 0.024 respectively). The antigen DQ1 was significantly decreased in T1DM patients (6.66%) *vs.* (22.5%) in controls with PF value of 0.168, such negative association (P=0.008) remained significant after correction (PC=0.024).

HLA Association with T1DM in Siblings

At HLA-class II region (DR loci), increased frequency of antigen DR4 (34.00 *vs.* 12.50%, P=0.003) was observed in the siblings. The RR value of such positive association was 2.428, and the EF value was 0.176. This association was significant (P=0.003) before correction, and after correction (PC=0.027). On the other hand DR5 antigen showed decreased frequency in the siblings as compared to controls (2.00 *vs.* 11.25% respectively) with PF value of 0.095. Such negative association was significant before correction (P=0.049) but not after (PC=0.441).

T1DM Patients *vs.* Siblings

As listed in table (2), both the T1DM patients and their siblings shared the HLA-DQ1 as protective antigens, while DR3 and DR4 were susceptible one. No other antigen in the present study was found to be common between the patients and their siblings. Such association was significant before correction but not after (PC= 0.152, 0.228 and 0.057 respectively)

Table 1: HLA antigen frequencies in control, T1DM patients and Siblings groups.

HLA-antigens	Control (Number = 80)		TIDM patients (Number =60)		Siblings (Number = 50)	
HLA-DR locus	**No.**	**%**	**No.**	**%**	**No.**	**%**
DR1	18	22.50	21	35.00	12	24.00
DR2	20	25.00	4	6.66	7	14.00
DR3	21	26.25	32	53.33	18	36.00
DR4	10	12.50	30	50.00	17.0	34.00
DR5	1	11.25	2	3.33	1	2.00
DR6	3	3.75	6	10.00	3	6.00
DR7	12	15.00	14	23.33	10	20.00
DR8	8	10.00	11	18.33	6	12.00
DR10	0	ND	4	6.66	6	12.00
HLA-DQ locus						
DQ1	18	22.5	4	6.66	11	22.00
DQ2	12	15.00	20	33.33	11	22.00
DQ3	16	20.00	24	40.00	15	30.00

ND: not detected

Table 2: Antigens of HLA-class II regions showing significant variations between T1DM patients, siblings and controls.

HLA	TIDM *vs.* control					Siblings *vs.* control					TIDM *vs.* siblings	
	RR	EF	PF	P	PC	RR	EF	PF	P	PC	P	PC
DR2	0.214	–	0.195	0.003	0.027	–	–	–	–	–	–	–
DR3	3.210	0.366	–	$9.7x10^{-3}$	0.008	–	–	–	–	–	0.051	NS
DR4	7.00	0.428	–	$1x10^{-5}$	$9x10^{-5}$	2.428	0.176	–	0.003	0.027	0.026	NS
DR5	–	–	–	–	–	0.160	–	0.095	0.049	NS	–	–
DQ1	0.246	–	0.168	0.008	0.024	–	–	–	–	–	0.019	NS
DQ2	2.833	0.215	–	0.009	0.027	–	–	–	–	–	–	–
DQ3	2.666	0.249	–	0.008	0.024	–	–	–	–	–	–	–

RR: relative risk; EF: Etiological fraction; PF: Preventive fraction; P: Fisher exact probability; PC: Corrected probability

GAD_{65} autoantibodies:

GADA were detected in 30 of Iraqi children with newly diagnosed T1DM (50%). The proportion of index cases positive in comparison with controls and siblings were shown in table (3). A higher significant proportion of the patients was positive to GADA (30/60, 50%) as compared to control groups (3/50; 6%), and siblings (8/50, 16%) This differences were highly significant between patients and controls(P_1=0.0001), patients and siblings (P2=0.0001), but not significant between siblings and controls (P3>0.05).

Table 3: Differences of sero positive /negative of GADA between control, T1DM patient and sibling groups.

Groups	No.	Sero positive		Sero negative		P_1	P_2	P_3
Controls	50	3	6.00	47	94.00			
T1DM	60	30	50.00	30	50.00	Chi 0.0001 (HS)	Chi 0.0001 (HS)	Chi 0.489 (NS)
Siblings	50	8	16.00	42	84.00			

P_1 : Patients *vs.* controls P_2 : patients *vs.* siblings
P_3: siblings *vs.* controls NS: Not significant

Relation of HLA-DR, -DQ Risky Alleles with Sero-Positive GADA in T1DM Patients

The HLA conferred susceptibility was graded into four categories:

No risk HLA **(**others**)**: non DR3/ non DR4; non DQ2/ non DQ3.

Low risk HLA: DR3/ non DR4; DQ2/ non DQ3.

Moderate risk HLA: DR4/ non DR3; DQ3/ non DQ2.

High risk HLA: DR3/ DR4; DQ2/ DQ3.

Table (4) represented the distribution of sero-positive GADA in patients with HLA-DR risky alleles, and in those with other different alleles. The proportion of sero-positive GADA in patients with HLA-DR3/DR4 high risk alleles were significantly higher (P= 0.001) than those who had other alleles. The DR3/DR4 combination seemed to have the high prevalence (53.33%) compared to DR4 (10.0%) and DR3 (6.67%).

Table 4: Distribution of sero-positive GADA in T1DM patients and relation with HLA-DR risky alleles.

Parameter	No.	DR3/DR4 No. (%)	DR3 No. (%)	DR4 No. (%)	Others No. (%)	P
GADA +ve	30	16 (53.33)	2 (6.67)	3 (10.0)	9 (30.0)	0.001 (S)

Chi = 16.523

The results represented in table (5) indicate a high proportion of $GADA^+$ in patients carrying DQ3 risky allele (43.33%) compared to DQ2/DQ3 (23.33%) and DQ_2 (10%). By using chi-square test, the statistical analysis showed a significant differences of sero-positive GADA in patients with DQ risky alleles than those carrying other alleles (P= 0.016).

Table 5: Distribution of sero-positive GADA in T1DM patients and relation with HLA-DQ risky alleles.

Parameter	No.	DQ2/DQ3 No. (%)	DQ3 No. (%)	DQ2 No. (%)	Others No. (%)	P
GADA +ve	30	7 (23.33)	13 (43.33)	3 (10.00)	7 (23.33)	0.016 (S)

Chi = 5.059

Relation of HLA-DR, -DQ Risky Alleles with Sero-Positive GADA in Siblings
Table (6) represented the distribution of sero-positive GADA in siblings with HLA-DR risky alleles, and in those with other different alleles The proportion of sero-positive GADA in siblings with HLA-DR risky alleles were not significantly different ($p>0.05$) than those who had other alleles, although the DR3/DR4 combination seemed to have the high prevalence (37.5%) compared to DR4 (25%) and DR3 (12.5%).

Table 6: Distribution of sero-positive GADA in Siblings and relation with HLA-DR risky alleles

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Parameter	No.	DR3/DR4 No. (%)	DR3 No. (%)	DR4 No. (%)	Others No. (%)	P
GADA +ve	8	(37.5)	1(12.5)	2(25.0)	2(25.0)	0.465 (NS)

The results represented in table (7) indicate a high proportion of $GADA^+$ in siblings carrying DQ2 risky allele (37.5%) and other alleles (37.5%). By using chi-square test, the statistical analysis showed no significant differences of sero-positive GADA in patients with DQ risky alleles than those carrying other alleles ($P> 0.05$).

Table 7: Distribution of sero-positive GADA in Siblings and relation with HLA-DQ risky alleles.

Parameter	No.	DQ2/DQ3 No. (%)	DQ3 No. (%)	DQ2 No. (%)	Others No. (%)	P
GADA +ve	8	1(12.5)	1(12.5)	3(37.5)	3(37.5)	0.157 (NS)

Discussions:

HLA Association Alleles

Type1diabetes mellitus can be considered as an organ-specific autoimmune disease. It is known that T1DM has been transferred from prediabetic subjects to an HLA identical sibling as a consequence of bone marrow transplantation (15). HLA showed different distributions in patients, siblings and controls such differences can be explained in the ground of racial differences, especially if we consider that HLA antigens show different frequencies in different populations including Iraqis, this indicates that this marker is partially involved in the disease development (16), and other factors like environment factors can be involved.

At HLA-class II region, many antigens had positive associations with T1DM. These were DR3, DR4, DQ2 and DQ3. However, multiple studies have reported association between HLA-DR and DQ phenotypes and

T1DM. DQ2.DR3 and DQ3.DR4 haplotypes reported as high risk alleles in Caucasians (17), while DR4, DQ4 were found to be dominant in Japanese (18). In Finland, DQ2/DQ3 genotype was found to be associated with genetic susceptibility and was more frequent in children diagnosed <5 years of age (19). Al-Samarria, reported high significant association of HLA-DR3, DR4, DQ2 and DQ3 with T1DM in Iraqi patients (20). Studies of HLA genes at the molecular levels showed that this association with HLA-DR is secondary to a stronger link with certain HLA-DQ variants. It is worthy to note that amino acid 57 in HLA-DQB lies in the "antigen binding groove". Antigens DR2 and DQ1 showed a negative association with the disease, these antigens may have protective effect.

In siblings, a significant increased frequency of antigen DR4 was observed in comparison with control subjects, and the positive association remains significant after correction. Concerning other world population studies, HLA-DR4 was found to be associated with the presence of ICA (7%) in siblings of T1DM Mexican-American patients (21). This locus is known to be associated with T1DM risk particularly with in type 1 diabetes families (22). Thus it may be much more useful for predicting T1DM in affected families than in population. Sheehy *et al.,* detected a highly diabetogenic subset of DR4 haplotypes among T1DM patient's sibling and he suggested that DR typing is 6-10 times less powerful as predictor of T1DM in the population than among patients siblings (23) Clearly, the structural differences seen between the predisposing and protective HLA molecules will affect their ability to bind or interact with diabetogenic antigens and the T- cell receptor (TCR) of autoreactive β-cell specific T-cells (24).

Anti-GAD Autoantibodies

GAD autoantigen is neither beta-cell nor islet specific and is expressed predominantly in the nervous system and other tissues, including the testes, ovary, adrenal, pituitary, thyroid and kidney (25). Islet cells reactivity as judged by the presence of antibodies to the GAD_{65} were observed in 50% of the patients studied and 16% of siblings (table 3).

The functional role of GADA in the pathogenesis of T1DM comes from their relationship to T-cell reactivity to GAD_{65} autoantigen. Presentation of an immunodominant T-cell epitope from the human GAD_{65} autoantigen is enhanced by GAD_{65} autoantibodies through increasing the efficiency of antigen capture by APCs including Fc receptor (FcR)-Positive monocytes/macrophages (26).

Relation of GADA with HLA

The results in table (4) indicated that GADA were found at the highest levels in index cases carrying DR3/DR4 heterozygous. This indicates that GADA expression is regulated genetically. It is known that there is an over-representation of DR3/DR4 heterozygous subjects among young children with newly diagnosed T1DM as compared with adolescents and adults with recent-onset disease (27). These observations support the concept that a strong genetic susceptibility is associated with aggressive rapidly progressing β-cell destruction as reflected by marked GADA responses and clinical manifestation of T1DM at young age, while a weaker genetic predisposition results in a slower destructive process and disease presentation in adults. In this study a low frequency of GADA is observed in the patients who were homozygous for DR3. In contrast, Sabbah, reported that increased GADA concentration was the charactistic of DR3/DQ2 haplotypes (8). Another study reported that only T-cell reactive with GAD-derived peptides in the context of DR-heterodimers could be isolated form the periphery of T1DM patients (28), indicating that HLA-DR rather than DQ seems to be the principle restriction element used by T-cells present at the onset of the disease.

The results represented in table (6) indicate a high proportion of $GADA^+$ in siblings carrying DR3/DR4 heterozygous risky allele (37.5%), although this proportion was not significantly different from other alleles. The high- risk HLA

DR3/DR4 genotype was associated with increased progression to type 1 diabetes (29). Achenbach, *et al.,* found that no striking stratification by HLA genotype or by multiple type 1 diabetes family history in progression to type 1 diabetes in relatives who have multiple islet auto antibodies (30). The lack of HLA- conferred disease susceptibility indicates that the pace of the pre- diabetic disease process is mainly regulated by factors other than the HLA class II genes (31), although positivity for only one type diabetes- associated autoantibody appears to reflect harmless and even reversible β- cell autoimmunity. HLA influences progression to T1DM and disease risk can be assigned to a given haplotype or genotype (32). However, it is controversal whether HLA influences the development of positive autoantibodies, the subsequent progression to T1DM, or both stages (31). Of note, among multiple antibody-positive relatives, progression to T1DM was not different by HLA genotype(33). Furthermore, the influence of most genotypes was not independent from the expression of auto antibodies. In relatives expressing multiple antibodies diabetes risk or protection was not further determined by HLA (33).

Conclusions:

The present study detected that immunogenetic predisposition may be considered as an important factor for the development of T1DM in association with the HLA antigens.

1. The HLA-class II (-DR3, DR4, DQ2 and DQ3) antigens were significantly increased in T1DM patients and may played an important role in the etiology of the disease, while DR2 and DQ1 antigens were significantly decreased in the patients. In siblings a significant increase was observed in HLA-DR4 antigen compared to control group, both the T1DM patients and their siblings shared the HLA- DQ1 as protective antigens, while DR3 and DR4 were susceptible one.
2. GADA were present in 50% of diabetic children and in 16% of their siblings. High proportion of GADA was found in the T1DM patients and siblings carrying HLA-DR3/DR4 heterozygous.

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